Thyroid cancer treatment

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CHAPTER 9

Increased global DNA hypomethylation in metastatic and dedifferentiated thyroid cancer

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In preparation

ABSTRACT

Introduction

Genetic and epigenetic alterations are crucial for the development and progression of cancer. One of these events is global DNA hypomethylation, of which the significance in thyroid cancer remains unclear. Therefore, we aimed to investigate whether global DNA hypomethylation plays a role in thyroid cancer progression and can act as a prognostic marker.

Methods

DNA was extracted from formalin-fixed paraffin-embedded tissues, as well as from fresh frozen tumors. Global hypomethylation of Alu elements (the most abundant repetitive elements of the human genome) was used as a surrogate marker for DNA global hypomethylation, and was assessed using the Quantification of Unmethylated Alu (QUAlu) technique.

Results

Primary tumors of 90 thyroid cancer patients were included ($n = 28$ low-risk differentiated thyroid cancer (DTC), $n = 13$ pediatric DTC, $n = 33$ distant metastatic DTC, $n = 7$ poorly differentiated (PDTC) and $n = 9$ anaplastic thyroid cancer (ATC)), as well as tissues from 20 distant metastases, and 20 normal thyroid tissues. An increasing hypomethylation was found for distant metastatic DTC (median 4.0, IQR 3.1 - 6.2) and PDTC/ATC tumors (median 9.3, IQR 7.0 - 12.1) as compared to normal thyroid tissue (median 2.75, IQR 2.30 - 3.15), $p < 0.001$ for both, whereas low-risk and pediatric DTC tumors were not affected by hypomethylation. Global Alu hypomethylation was similar between distant metastatic tissues and matched primary tumors. Kaplan-Meier and unadjusted and age-adjusted Cox regression analyses showed that thyroid cancer-related and all-cause mortality were related to tumor hypomethylation, but this association was lost after further adjustment for thyroid cancer risk category.

Conclusion

Metastatic DTC, PDTC and ATC tumors were increasingly affected by global Alu hypomethylation, which suggests that this epigenetic entity may be involved in thyroid cancer progression and dedifferentiation.

INTRODUCTION

Thyroid cancer is an increasingly common malignancy, which is especially true for differentiated thyroid cancer (DTC).[1] In particular patients with low-risk DTC and pediatric DTC have an excellent prognosis, although the latter generally present with rather extensive disease.[2,3] However, for patients with DTC who develop distant metastases or radioiodine-refractory disease, the prognosis is poor,[4] which is the case as well for patients with poorly differentiated (PDTC) and anaplastic thyroid carcinoma (ATC).[5] When initial treatment fails in these high-risk thyroid cancer patients, an effective treatment is currently lacking. The key for the future development of treatments probably lies in an improved understanding of the molecular events in tumors of DTC patients with distant metastatic disease, as well as in patients with PDTC and ATC.

Genetic and epigenetic alterations are critical players in cancer development and progression.[6] Several genetic alterations that affect the MAPK and PI3K/AKT pathways have been found in DTC.[7,8] Of these, mutations in BRAF and RAS and rearrangements in RET/PTC and PAX8/PPARG are the most common for DTC. Furthermore, an increasing amount of data is becoming available about epigenetic modifications in thyroid cancer, especially DNA methylation.[9-11] DNA methylation is one of the most well-characterized epigenetic entities which consists of the addition of a methyl group to a cytosine nucleotide. In mammals this mainly occurs in cytosines that precede a guanine (a so called CpG site), and constitutes an inactive mark associated with transcriptional inactivation and repressed chromatin. The human genome is largely methylated, except for CpG-rich regions called CpG islands that are mostly located in gene promoter regions.[12,13] In cancer, two main alterations in DNA methylation have been distinguished.[14] On the one hand, it is common to find locus-specific hypermethylation that mainly affects regulatory elements such as CpG islands in promoter regions, which can lead to silencing of tumor suppressor genes or genes that are important for cellular function (for example DNA repair and apoptosis).[15] On the other hand, DNA hypomethylation can be found, which affects extensive domains of the genome, and promotes genomic instability. Especially repetitive DNA elements such as Alu repeats and long-interspersed nuclear elements-1 (LINE-1) are altered by global DNA hypomethylation.[16] Interestingly, an increasing body of evidence shows an association between loss of DNA methylation and the early stages of tumorigenesis or tumor progression, and therefore it has been proposed as a cancer biomarker.[17-19]

Nearly half of the human genome is comprised of repetitive sequences, Alu repeats being the most abundant.[20] Alu elements are primate-specific transposons (i.e. they can move within the genome), and make up almost 11% of the genomic mass. Approximately 25% of all human CpG sites are located within Alu repeats and most of them are methylated. However, a fraction remains unmethylated and this proportion is increased in cancer.[21] Alu methylation levels have been shown to correlate well with global DNA methylation, and could therefore be used as a surrogate marker for global hypomethylation.[22] In this regard, several studies found a global Alu hypomethylation in different cancer types.[18,23-25]

Since the role of global DNA hypomethylation remains unclear in progressive thyroid cancer, we aimed to investigate whether it is increased in primary tumors of patients with low-risk and distant metastatic DTC, pediatric DTC, and PDTC and ATC, using the recently developed **Q**uantification of **U**nmethylated **Alu** (QUAlu) technique.[25] Furthermore, we aimed to assess whether global Alu hypomethylation is

altered in distant metastases as compared to their primary tumors, and whether it can act as a marker to differentiate between patients at risk for thyroid cancer-related and all-cause mortality.

MATERIALS AND METHODS

Patients and samples

We analyzed tumor tissue of patients with low-risk DTC (defined as patients with well-differentiated papillary (PTC), or follicular (FTC) thyroid cancer with tumor stage T1-2, Nx-N1, M0, who were disease-free after initial treatment, and remained disease-free during a follow-up of at least 5 years), and distant metastatic DTC (defined as any PTC or FTC patients with distant metastases at presentation or during follow-up). Furthermore, we included tumor tissues from patients with childhood-onset PTC, PDTC, and ATC. The study was accepted by the ethics committees of the participating centers (see supplemental methods). All patients who were still alive during sample recruitment provided written informed consent for use of their thyroid tissue.

Primary tumors, and, if available, tissues from corresponding distant metastases (mostly from bone or lung) were obtained after examination by an experienced pathologist. Furthermore, several paired normal thyroid tissues were analyzed. Formalin-fixed paraffin-embedded (FFPE) tissues were used for patients with DTC. For patients with ATC and PDTC, fresh frozen tissues were considered as well. For each FFPE sample, 10 non-stained slices of 10 µm thickness (containing at least 80% tumoral cells) were cut from the paraffin blocks. Thereafter, FFPE samples were deparaffinized using xylene, and genomic DNA was extracted with E.Z.N.A. FFPE DNA-Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. For fresh frozen ATC and PDTC tissues, the DNeasy Blood and Tissue kit (QUIAGEN, Valencia, CA, USA) was used. All DNA samples were quantified by a fluorometric method (Qubit, Thermo Fisher Scientific). DNA purity was assessed by NanoDrop (Thermo Scientific) and the integrity was checked by electrophoresis in a 1% agarose gel.

Cell lines

Nine thyroid cancer cell lines were analyzed: 2 PTC-derived (TPC1, KTC-1), 2 FTC-derived (FTC133, WRO82-1) and 5 ATC-derived (CAL-62, SW1736, 8505C, HTh7, HTh83). Genomic DNA (provided by Dr. M. Robledo) was extracted using the DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol.

The colorectal carcinoma cell line HCT116 was obtained from the American Type Culture Collection. Cells were cultured in D-MEM/F12, supplemented with sodium pyruvate, L-glutamine and 10% fetal bovine serum (Life Technologies, MD, USA) and were maintained at 37ºC in a 5% CO_2 atmosphere. DNA was extracted using the PureLink Genomic DNA kit (Invitrogen, Carlsbad, CA, USA). To prepare the FFPE cell block, 50 x 10^6 HCT116 cells were pelleted by centrifugation, fixed with formaldehyde overnight and embedded in paraffin blocks.

QUAlu

As previously described in detail,[25] the QUAlu technique allows the relative quantification of unmethylated Alu repeats. It relies on the amplification of Alu elements with an umethylated CpG site within the consensus Alu sequence AACC**CG**G. First, DNA was digested in separate tubes (both containing 5 ng of DNA) using the methylation sensitive and insensitive restriction enzymes *Hpa*II and *Msp*I, respectively, whose restriction site is C/CGG. *Hpa*II solely cuts unmethylated CpG sites, whereas *Msp*I cuts these sites irrespective of methylation status. Both enzymes leave an identical sticky end, to which a synthetic adaptor was subsequently ligated. The last step consisted of amplification of the *Hpa*II and *Msp*I digested-ligated DNA (1:20 diluted) by quantitative PCR (qPCR) using a reverse primer homologous to the adapter and a forward primer specific for the Alu consensus sequence. Furthermore, we performed a specific qPCR to amplify L1PA (a long interspersed nuclear elements-1 (LINE-1) subfamily), as internal control to normalize DNA input. This finally allowed the calculation of the Percentage of UnMethylated Alu elements (PUMA) for each sample.

Study definitions

For all patients clinical data were obtained, i.e. baseline characteristics (sex, age at diagnosis, tumor histology, TNM classification), treatment characteristics (surgery, radioiodine treatments and dose, use of tyrosine kinase inhibitors), and survival data. Follow-up time was defined as the time between the date of thyroid cancer diagnosis and the date of the last follow-up record or death.

Statistical analysis

Data were presented as number (percentage), median (inter quartile range (IQR)), or mean±standard deviation (SD), as appropriate. The PUMA was calculated (as previously described[25]) as the ratio between quantifiable unmethylated Alu elements (normalized for L1PA levels of *Hpa*II digested DNA) and total amount of amplifiable Alu elements (normalized for L1PA of *Msp*I digested DNA). Permutation tests were performed using R and the qpcR package (v1.4-0),[26] to obtain the final PUMA. PUMA was compared between normal thyroid tissues and several thyroid cancer risk categories (low-risk DTC, pediatric PTC, distant metastatic DTC, and PDTC/ATC) using the non-parametric Mann Whitney U test. The Wilcoxon signed rank test was applied to test differences between PUMA of paired normal thyroid and tumor tissues. A thyroid tumor was considered hypomethylated when the PUMA was above the 99th percentile of PUMA of normal tissues,[25] which was 4.3%. Correlation analyses were performed using Spearman's Rho. Kaplan-Meier and unadjusted and adjusted Cox regression analyses were performed to assess the relation between PUMA and thyroid cancer-related and all-cause mortality.

A p-value < 0.05 was considered statistically significant. Analyses were performed using SPSS (version 22.0), and R (version 3.2.2).

RESULTS

Patient characteristics

We included 90 patients with thyroid cancer for this study. A total of 28 patients had low-risk DTC, 13 pediatric PTC, 33 distant metastatic DTC, 7 PDTC and 9 were diagnosed with ATC. See Table 1 for baseline and tumor characteristics, and Table 2 for an overview of the treatments administered, and the survival data.

Table 1 | Baseline and tumor characteristics.

Parameter	Low-risk DTC	M1 DTC	Pediatric PTC	PDTC/ATC
n	28	33	13	16
Age mean±SD	45.1 ± 13.6	59.5 ± 14.2	14.8 ± 2.8	67.8 ± 15.4
Female sex *n* (%)	23 (82.1)	25 (75.8)	11 (84.6)	10 (62.5)
Histology *n* (%)				
PTC	20	20	13	
FTC	8	13		
ATC				9
PDTC				7
TNM classification *n* (%)				
T stage				
T1	10	5	4	0
T2	18	6	2	2
T3	0	10	5	4
T4	0	12	2	10
N stage				
Nx-N0	19	16	6	5
N1	9	17	7	11
M stage				
Mx-M0	28	0	10	7
M1	0	33	3	9
M1 site				
Bone	0	8	0	0
Lung	0	11	3	1
Multiple sites	0	9	0	2
Other	0	1	0	0
Unknown	0	4	0	6

Abbreviation: M1 DTC = distant metastatic DTC

Technical evaluation of QUAlu in FFPE tissues

The QUAlu technique has previously been applied to a wide range of different clinical biospecimens,[25] but it has not been extensively examined in FFPE tissues. Using different starting amounts of FFPE tissue-derived DNA (ranging from 0.3 to 80 ng), we obtained an excellent linear response ($R^2 > 0.95$) in Cq values (quantification cycle values, or number of PCR cycles needed to exceed the background level, i.e. the lower the Cq value, the more DNA is present) in the different qPCRs (Figure 1). This indicates that the QUAlu technique can be applied to a wide variety of DNA input amounts, including very low quantities. Additionally, we analyzed the colon cancer cell line HCT116 using DNA from fresh frozen cells and from a FFPE cell block, and found similar PUMAs. Moreover, PUMA was equal in DNA from normal thyroid tissues derived from fresh frozen and FFPE tissues (median [IQR] PUMA 2.9 [2.7 - 3.2] and 2.8 [2.3 - 3.2], respectively, $p = 0.307$).

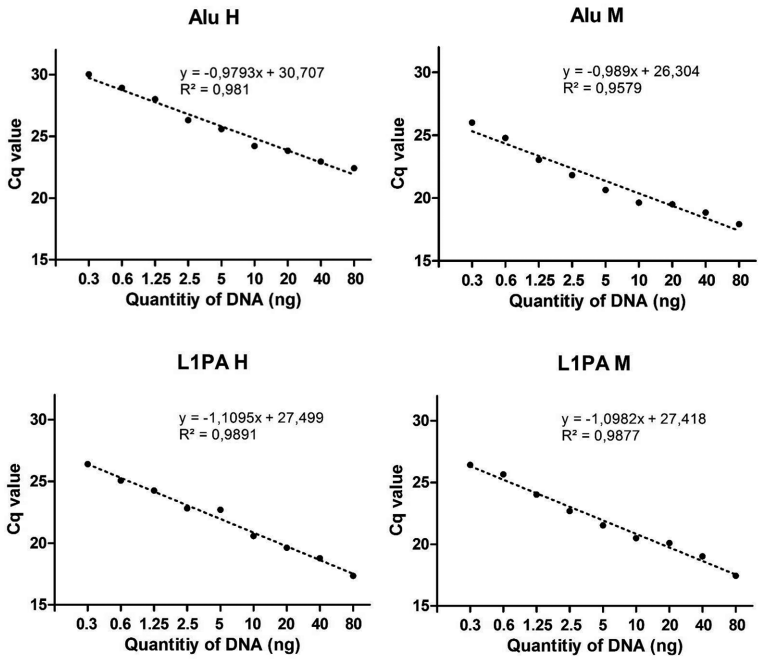

Figure 1 | Standard curves of Alu and L1PA qPCR data from HpaII (H) and MspI (M) digested DNA. Using quantities of DNA ranging from 0.3 to 80 ng as starting material during the QUAlu method, a linear response in quantification cycle (Cq) values was obtained. DNA extracted from a FFPE thyroid tumor was used for these analyses.

Global DNA hypomethylation of thyroid tumors

The QUAlu technique was applied to a series of normal thyroid tissues and primary and distant metastatic tumors of thyroid carcinomas (Figure 2 and Table 3). The PUMAs of normal tissues ($n = 20$) were homogeneous (median 2.75, IQR 2.30 - 3.15), and these were similar to the PUMAs of primary low-risk DTC tumors (median 2.9, IQR 2.6 - 3.5, $p = 0.336$). In contrast, primary tumors of patients with distant

metastatic DTC had a significantly higher PUMA as compared to normal tissues (median 4.0, IQR 3.1 - 6.2), as well as patients with PDTC/ATC tumors (median 9.3, IQR 7.0 - 12.1), both $p < 0.001$. In accordance, the PUMA was equal between normal thyroid and matched primary tumor tissues of low-risk DTC ($p = 0.784$), whereas the PUMA of thyroid cancer tumor tissues of patients with distant metastatic DTC was higher as compared to the matched normal thyroid tissues ($p = 0.018$) (Figure 3). The PUMA of pediatric PTC patients was median 3.2 (IQR 2.9 - 3.6), which was not statistically different from the PUMA of normal thyroid tissue of adult patients ($p = 0.057$).

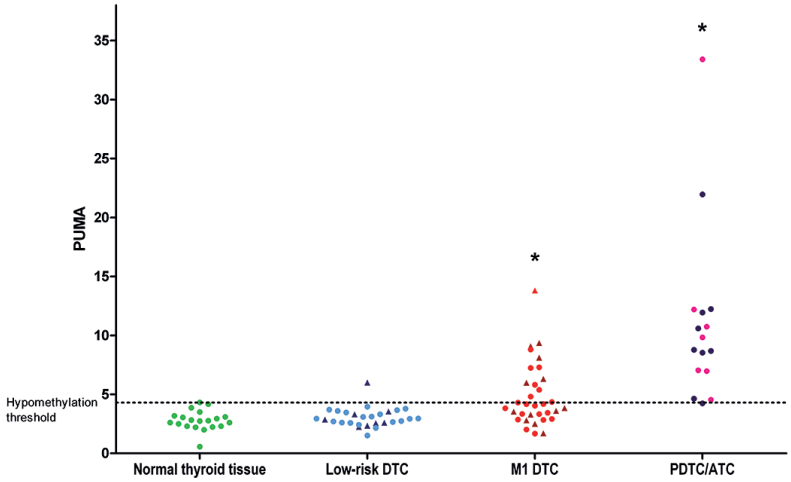

Figure 2 | PUMA of normal thyroid tissue and primary thyroid cancers, represented by thyroid cancer risk categories. The darker triangle-shaped points within DTC categories represent patients with FTC, the lighter points represent PTC patients. Within the PDTC/ATC category, the darker and lighter points represent ATC and PDTC patients, respectively. * $p < 0.001$ relative to normal thyroid tissues. The hypomethylation threshold (defined as the 99th percentile of normal thyroid tissues) is indicated by a horizontal dashed line. *Abbreviation:* M1 DTC = distant metastatic DTC.

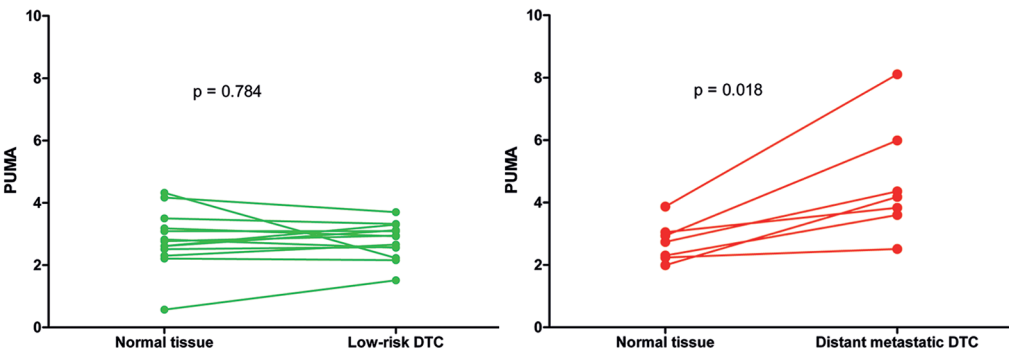

Figure 3 | PUMA of paired normal thyroid tissues and primary tumors of low-risk DTC (left panel), and distant metastatic DTC (right panel).

Table 2 | Administered treatment and survival data.

Parameter	Low-risk DTC	M1 DTC	Pediatric PTC	PDTC/ATC
n	28	33	13	16
Initial surgery *n* (%)				
Total thyroidectomy	27 (96.4)	33 (100)	13 (100)	3 (18.8)
Hemi-thyroidectomy	1 (3.6)	0	0	0
Unknown	0	0	0	13 (81.3)
Radioiodine treatment *n* (%)				
Ablation therapy				
Yes	26 (92.9)	31 (93.9)	13 (100)	6 (37.5)
No	1 (3.6)	1 (3.0)	0	2 (12.5)
Unknown	1 (3.6)	1 (3.0)	0	8 (50.0)
Ablation dose (mCi)				
Median [IQR]	125 [100 - 125][a]	150 [150 - 150][b]	100 [50 - 150]	150 [0 - 150][c]
Cumulative dose (mCi)				
Median [IQR]	125 [100 - 125][a]	300 [150 - 450][a]	299 [66 - 375]	150 [120 - 150][d]
Tyrosine kinase inhibitors *n* (%)	0	9 (27.3)	0	0[e]
Mortality *n* (%)				
All-cause mortality	1 (3.6)	17 (51.5)	0	11 (68.8)
Thyroid cancer mortality	0	15 (45.5)	0	11 (68.8)
Follow-up time (years)				
Median [IQR]	9.0 [7.0 - 11.0]	5.0 [3.0 - 7.1]	8.4 [2.4 - 13.1]	0.9 [0.4 - 2.9]

Data are missing for [a] 1, [b] 6, [c] 11, [d] 9, [e] 13 patients. *Abbreviation:* M1 DTC = distant metastatic DTC

The percentages of hypomethylated tumors (i.e., a PUMA > the 99th percentile of normal tissues) are shown by thyroid cancer risk category in Table 3. Tumors of patients with low-risk DTC and pediatric PTC were rarely hypomethylated, whereas distant metastatic DTC tumors were hypomethylated in 42% of cases. PDTC and ATC tumors were highly hypomethylated, in 94% of cases.

When available, we assessed the PUMA of distant metastatic tissues as well. The PUMAs of distant metastases (n = 20 tissues, from 15 patients) were similar to the PUMA of the matched primary thyroid tumor (p = 0.893) (Figure 4).

Furthermore, we quantified the PUMA of different thyroid cancer cell lines. All of them were highly hypomethylated in comparison to normal thyroid tissue. The PTC-derived cell lines had an average PUMA of 13, while the FTC- and ATC-derived cell lines had an average of 32 and 22, respectively (Figure 5).

Table 3 | Percentage of unmethylated Alu repeats (PUMA) and tumor hypomethylation status represented by thyroid cancer risk category.

	Low-risk DTC	M1 DTC	Pediatric PTC	PDTC/ATC
n	28	33	13	16
PUMA				
median [IQR]	2.9 [2.6- 3.5]	4.0 [3.1 - 6.2]	3.2 [2.9 - 3.6]	9.3 [7.0 - 12.1]
Tumor hypo-methylation *n* (%)	1 (3.6)	14 (42.4)	1 (7.7)	15 (93.8)

Abbreviation: M1 DTC = distant metastatic DTC

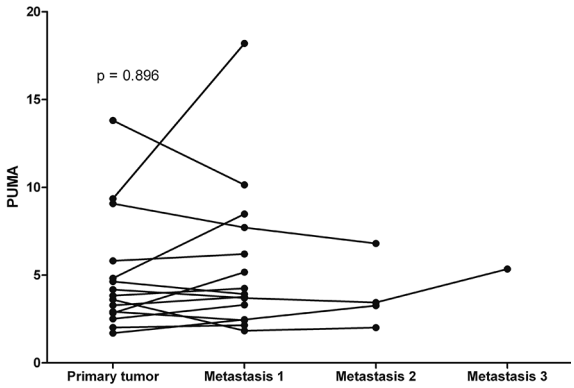

Figure 4 | Percentage of unmethylated Alu repeats (PUMA) of primary tumor tissues and matched distant metastases.

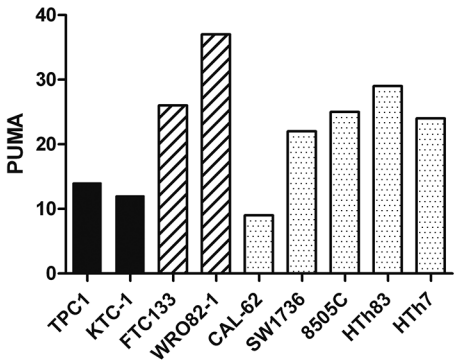

Figure 5 | Percentage of unmethylated Alu repeats (PUMA) of PTC-derived (black), FTC-derived (striped) and ATC-derived (dotted) cell lines.

PUMA and clinical data

Within normal thyroid tissues, PUMA did not correlate with age ($p = 0.762$). Conversely, in tumor tissues PUMA was correlated with age ($p < 0.001$), but after separate analyses in strata of low-risk DTC, pediatric PTC, distant metastatic DTC, and PDTC/ATC, this association was lost, indicating that PUMA is associated with thyroid cancer risk category rather than age.

Kaplan-Meier analyses showed that the endpoints thyroid cancer-related and all-cause mortality were affected by the hypomethylation state of the primary tumor (log-rank $p < 0.001$ for both, Figure 6). In unadjusted Cox regression analyses, each percent increase in PUMA was associated with a hazard ratio (HR) of 1.11 (95% CI 1.06 - 1.15) for both mortality endpoints. This association remained significant after adjustment for age, but was lost after further adjustment for thyroid cancer risk category: HR (95% CI) 1.02 (0.95 - 1.09) and 1.02 (0.96 - 1.10) for thyroid cancer-related and all-cause mortality, respectively.

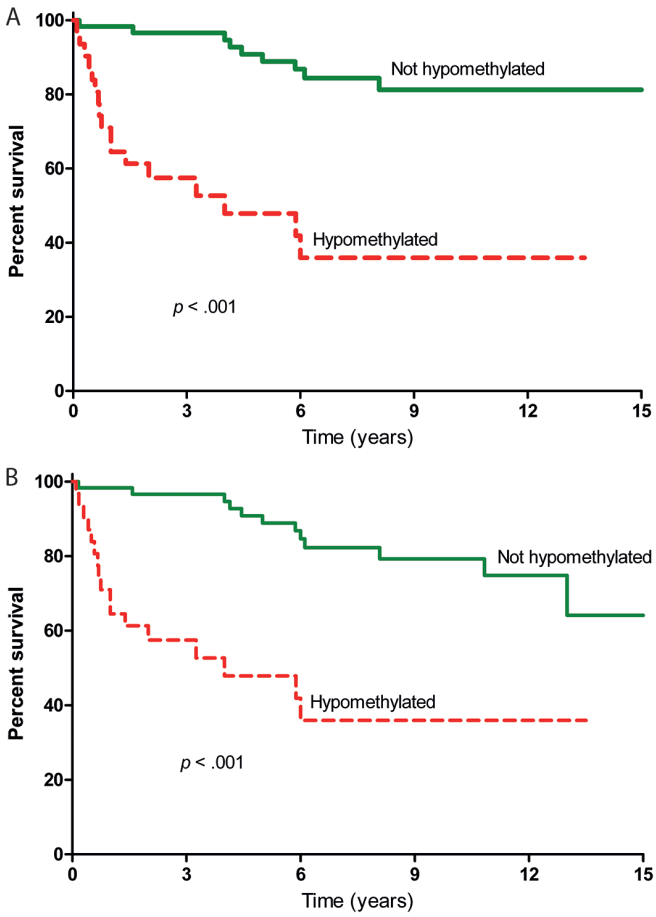

Figure 6 | Kaplan-Meier survival curve of (**A**) thyroid cancer-related survival and (**B**) all-cause mortality, by methylation status of the primary tumor (red dashed line = hypomethylated, green line = not hypomethylated).

Table 4 | Unadjusted and adjusted Cox regression models for the endpoints thyroid cancer-related and all-cause mortality by percentage of unmethylated Alu repeats (PUMA).

	Thyroid cancer-related mortality		All-cause mortality	
Cox regression model	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Unadjusted				
PUMA, per 1% increase	1.11 (1.06 - 1.15)	< 0.001	1.11 (1.06-1.15)	< 0.001
Age-adjusted				
PUMA, per 1% increase	1.07 (1.01 - 1.13)	0.014	1.07 (1.01-1.13)	0.015
Age- and risk category-adjusted*				
PUMA, per 1% increase	1.02 (0.95 -1.09)	0.637	1.02 (0.96-1.10)	0.505

Abbreviation: HR = hazard ratio, CI = confidence interval, * adjusted for age and thyroid cancer risk category

DISCUSSION

One of the hallmarks of cancer is global DNA hypomethylation. In the current study we assessed global Alu hypomethylation as a surrogate marker for global DNA hypomethylation in a broad prognostic spectrum of thyroid cancers, and found that Alu hypomethylation was increasingly affected in distant metastatic DTC, PDTC and ATC tumors. Conversely, hypomethylation was not increased in low-risk DTC and pediatric PTC tumors, and no further hypomethylation was observed in distant metastatic tissues as compared to the matched primary tumors.

DNA global hypomethylation in thyroid cancer has been assessed in several studies,[25,27-31] but these showed conflicting results with regard to the presence of global DNA hypomethylation. This can be explained by the low number of tumor samples that have been included in these studies, the poor characterization of thyroid tumors, and the usage of different techniques for assessment of global DNA hypomethylation. Here we analyzed a large series of thyroid tumors in a broad prognostic range. To the best of our knowledge this is the first study in which global Alu hypomethylation is assessed in thyroid tumors of pediatric DTC patients, and patients with distant metastatic DTC, PDTC and ATC. We used the recently developed QUAlu technique, which quantifies global Alu hypomethylation, and showed that it is easily applicable to a wide range of DNA quantities derived from FFPE tissues, including very low amounts of partly degraded DNA that is often encountered in pathological examination specimens. Furthermore, we confirmed that results were comparable to those from fresh frozen tissues since similar PUMAs were obtained in DNA derived from FFPE and fresh frozen samples.

Our findings revealed that global hypomethylation of Alu repeats seems to be a late event in thyroid cancer, as it occurred in 42% of distant metastatic DTC, and in most PDTC and ATC tumors, whereas it was virtually absent in low-risk DTC. Timp et al[31] showed that large hypomethylated genome blocks were a very early event being present in benign thyroid lesions and follicular adenomas, but this apparent discordance may be explained by the methodology they used which covers unique sequences but not repetitive elements.

Similar to our results, Alu hypomethylation was more pronounced in higher-grade prostate cancers,[24] whereas Alu hypomethylation was found to be implicated in early stage chronic lymphocytic leukemia,[18] and has been shown to be a very early event in tumorigenesis in colon carcinoma.[32] These results indicate that the pattern of Alu hypomethylation is cancer type-dependent.

Within DTCs, PTC and FTC tumors had a similar behaviour with regard to global Alu hypomethylation. In a prior study we found that FTC tumors had higher levels of methylation than PTC,[9] but only unique sequences within promoter regions were analysed, which probably explains the different results.

The increasing Alu hypomethylation in distant metastatic DTC, PDTC and ATC suggests that global DNA hypomethylation might be implicated in tumor progression or cell dedifferentiation. This is further illustrated by the PUMA values of the cell lines analyzed. These values fitted best in the range of PDTC and ATC tumors, which corresponds to the finding that thyroid cancer cell lines (either derived from PTC, FTC or ATC) show characteristics of dedifferentiated cells (with a gene expression resembling that of undifferentiated tumors, absence of thyroid-specific gene expression, and loss of TSH sensitivity).[33] However, it remains unknown whether global DNA hypomethylation acts as an oncogenic driver, or is a consequence of the overall genome deregulation in cancer. The prior was suggested in a mouse model in which induction of hypomethylation led to chromosomal instability and tumor development.[34] Furthermore, a direct link between DNA hypomethylation and genomic instability as well as poor survival has been reported in human colorectal cancer.[35,36]

We did not find an increased Alu hypomethylation in distant metastatic thyroid tissue as compared to the matched primary tumor, which implies that tumor hypomethylation remains relatively stable during distant metastatic spread in thyroid carcinoma patients. Likewise, in colorectal cancer patients similar global methylation levels were found in LINE-1 (a family of repetitive elements) in primary tumors and matched distant metastases,[37,38] although another study showed an increased LINE-1 hypomethylation in tissue from distant (liver) metastases as compared to the primary colorectal tumor,[39] which was the case as well for small intestinal neuroendocrine tumors.[40] These data point towards a cancer-specific involvement of global hypomethylation in distant metastatic spread.

Several studies from the last three decades have clearly demonstrated that aging, similar to cancer, is associated with a genome-wide decrease of DNA methylation.[41] To exclude that the association we found between thyroid cancer and global hypomethylation was an age-related event, we assessed the correlation between age and PUMA within thyroid cancer risk category strata. Here, no significant correlations were found between hypomethylation and aging. This indicated that global Alu hypomethylation was dependent upon risk classification rather than age, although we cannot exclude a significant association between age and PUMA within thyroid cancer risk categories due to a low statistical power. Pediatric DTC tumors showed a similar hypomethylation as adult normal thyroid tissues, which could reflect the well-differentiated character of these tumors.[2] Therefore, Alu hypomethylation does not seem to play an important role in the relative aggressiveness of pediatric PTC. However, we cannot preclude that lower cutoff levels for hypomethylation should be applied for pediatric patients. Furthermore, we found that Alu hypomethylation of primary thyroid tumors was associated with both thyroid cancer-related and all-cause mortality in unadjusted and age-adjusted Cox regression analyses. However, the association was lost after adjustment for thyroid cancer risk category, which precludes the use of PUMA as an independent biomarker for these endpoints in clinical practice.

In addition to Alu repeats, global DNA hypomethylation is commonly assessed in LINE-1 as a marker in cancer.[42] Although both LINE-1 and Alu repeats are highly repetitive sequences that comprise a substantial part of the genomic mass, hypomethylation of these elements is not necessarily the same in a tumor. Again, there may be a cancer-specific hypomethylation profile of repetitive elements. Similar global methylation profiles were for example found for Alu repeats, LINE-1 and α-satellite DNA in early stage chronic lymphocytic leukemia,[18] whereas methylation profiles of Alu repeats and LINE-1 were found to be different in several stages of breast cancer.[43] We chose to study Alu repeats for since these elements contain 25% of all CpG sites, are mostly located in gene-rich regions, and are relatively short elements that can be easily assessed using the QUAlu technique, even in FFPE samples that contain partly degraded DNA. Nonetheless, in further studies it would be interesting to additionally evaluate LINE-1 hypomethylation in different stages of thyroid cancer.

In conclusion, we found an increasing Alu hypomethylation in distant metastatic DTC, PDTC and ATC tumors, whereas low-risk DTC and pediatric PTC tumors were not affected by hypomethylation. This might reflect the involvement of global hypomethylation in a subset of thyroid cancers and its association with advanced disease and cell dedifferentiation. Alu hypomethylation seems to be rather stable during distant metastatic spread. Hence, DNA hypomethylation appears to be a new player in thyroid cancer. New studies are warranted to clarify its contribution to malignant behaviour, the interplay with other genetic and epigenetic alterations, as well as its potential application in preoperative fine needle aspiration cytology as a diagnostic and/or prognostic marker.

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